Exercise 5, Differentiation and colistin tolerance in *Pseudomonas aeruginosa* biofilms.

Introduction

Although the growth of bacteria in planktonic culture has been the mainstay of microbiological technique from the time of Pasteur to the present, and has provided an increasingly accurate understanding of prokaryotic physiology and genetics, it is now clear that bacteria in natural, industrial and clinical settings live in surface-associated communities, and that knowledge about this lifestyle is essential in order to understand bacterial biology in nature and disease. Such structured communities of cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface are termed biofilms.

One of the most important aspects of microbial biofilm biology is that the bacteria in these sessile communities display a remarkable increased tolerance to antimicrobial attack. Because of their innate resistance to host immune systems, antibiotics and other biocides, biofilms in medical and industrial settings are difficult, if not impossible, to eradicate. Biofilm formation therefore leads to various persistent and sometimes lethal infections in humans and animals, and to a variety of problems in industry where solid-water interfaces occur. Detailed knowledge of the developmental process from single cells scattered on a surface to the development of antimicrobial-tolerant biofilms is essential in order to create strategies to control biofilm development.

Structural biofilm development by *P. aeruginosa* appears to be conditional. For example, in flow-chambers irrigated with citrate minimal medium *P. aeruginosa* forms a flat biofilm, while in flow-chambers irrigated with glucose minimal medium it forms a heterogeneous biofilm with mushroom-shaped multicellular structures.

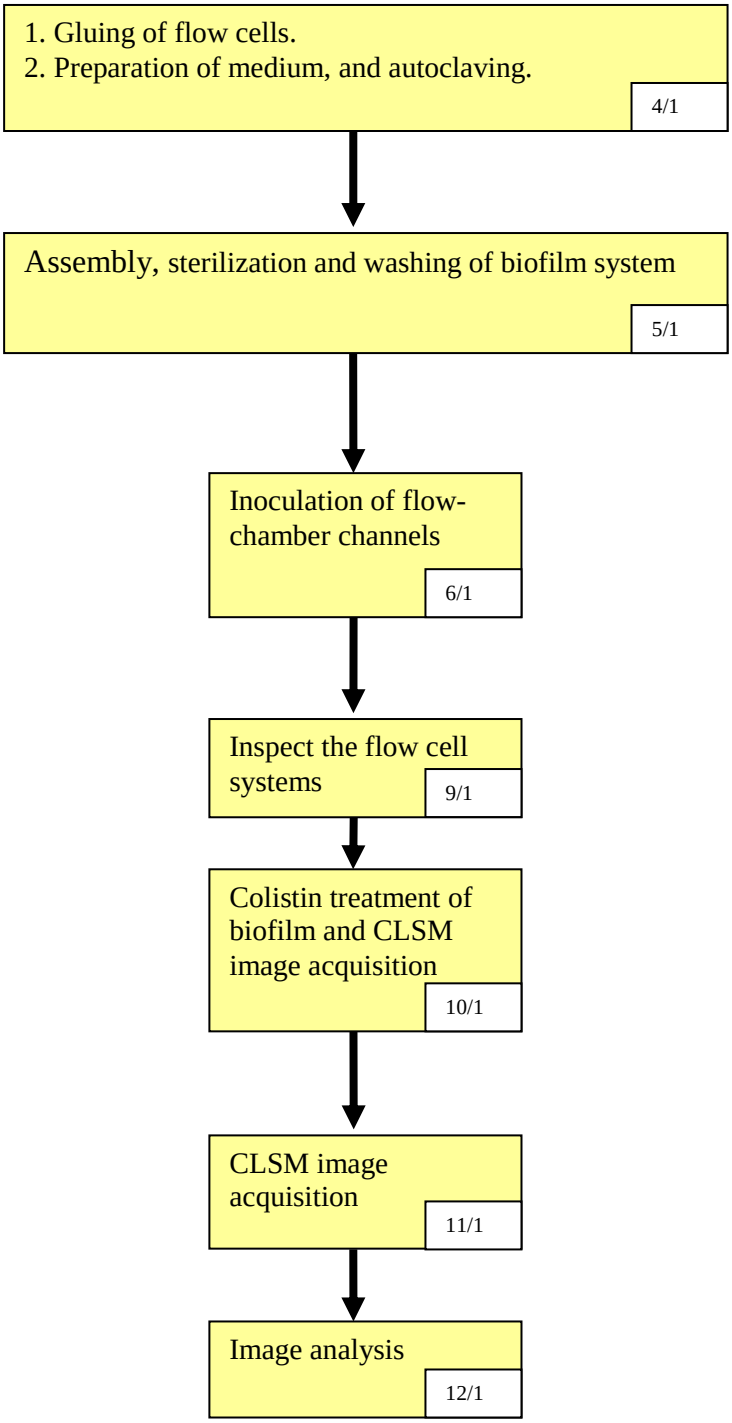
The formation of the flat *P. aeruginosa* biofilm in flow-chambers irrigated with citrate minimal medium was shown to occur via initial formation of microcolonies by clonal growth of sessile bacteria at the substratum, followed by expansive migration of the bacteria out on the substratum, resulting in the formation of a very dynamic flat biofilm. Since biofilm formation by a *P. aeruginosa pilA* mutant (which is deficient in biogenesis of type IV pili) occurred without the expansive phase, and resulted in discrete protruding microcolonies, it was suggested that the expansive migration of the bacteria out on the substratum was type IV pili-driven.

The formation of the mushroom-shaped structures in the glucose-grown *P. aeruginosa* biofilm was shown to occur in a sequential process involving a non-motile bacterial sub-population which form the initial microcolonies by growth in certain foci of the biofilm, and a migrating bacterial sub-population which initially form a monolayer on the substratum, and subsequently form the mushroom caps by climbing the initial microcolonies (which then become mushroom stalks) and aggregating on their tops via a process which is dependent on type IV pili. Development of the heterogeneous *P. aeruginosa* biofilm therefore evidently involves differentiation of the bacteria into at least two subpopulations (a motile and a non-motile).

Aims

In the present exercise the motile and non-motile subpopulations in glucose-grown *P. aeruginosa* biofilms, which forms cap and stalk of the biofilm mushrooms, are investigated with respect to tolerance to the antibiotic colistin and with respect to growth activity.

Exercise 5


1. Gluing of flow cells.
2. Preparation of medium, and autoclaving.
4/1
Assembly, sterilization and washing of biofilm system
5/1
Inoculation of flow-chamber channels
6/1
Inspect the flow cell systems
9/1
Colistin treatment of biofilm and CLSM image acquisition
10/1
CLSM image acquisition
11/1
Image analysis
12/1

Experimental procedures

Day 3 (4/1-11). Gluing of flow-cells, preparation of medium and autoclaving.

See the protocol for biofilm work on the wiki page:

Course manuals -> supplementary manuals and recipes -> general -> Biofilm general manual

Day 4 (5/1-11). Assembly of the biofilm system.

See the protocol for biofilm work on the wiki page:

Course manuals -> supplementary manuals and recipes -> general -> Biofilm general manual

Day 4 (5/1-11). Sterilization and washing of the biofilm system; saturate tubings with media over night.

See the protocol for biofilm work on the wiki page:

Course manuals -> supplementary manuals and recipes -> general -> Biofilm general manual

Two 16 channel setups should be prepared with ABT (+0.3 mM Glucose) medium

The strains will be inoculated in 10mL ABT (+ 30mM glucose) with appropriate antibiotics and incubated O.N. at 30°C. These cultures will be used to inoculate biofilms on day 5.

Day 5 (6/1-11). Inoculation of flow-chamber channels.

- See the protocol for biofilm work on the wiki page.

The following 'over night'(ON)-cultures will be provided:

6 x 10mL ABT (+ 30mM glucose) tubes containing respectively:

P. aeruginosa PAO1, miniTn*7*::*gfp* (Gentamicin resistant)

P. aeruginosa PAO1, miniTn*7*::*yfp* (Gentamicin resistant)

P. aeruginosa pilA, miniTn*7*::*cfp* (Streptomicin resistant)

P. aeruginosa PAO1 ::P_{rrn}-*gfp*(ASV) (Gentamicin resistant)

P. aeruginosa PAO1 ::P_{rrn}-*gfp*(AGA) (Gentamicin resistant)

P. aeruginosa PAO1 ::P_{rrn}-*gfp*(AAV) (Gentamicin resistant)

The ON cultures should be diluted 1000 times in 0.9% NaCL before inoculation. Each team inoculates **three** flow chamber channels: The first with *P. aeruginosa* PAO1,miniTn*7*::*gfp*, the second with *P. aeruginosa* PAO1::P_{rrn}-*gfp*(ASV)/(AGA)/(AAV), and the third with a 1:1 mixture of *P. aeruginosa* PAO1,miniTn*7*::*yfp* and *P. aeruginosa pilA*,miniTn*7*::*cfp*. Make sure all teams are ready, so that the flow can be started for all the channels at the same time.

Day 8 (9/1-11) Inspect the flow cell systems

Have the cells attached to the surface?

Day 9 (10/1-11). Colistin treatment

The 4 day-old PAO1 biofilm tagged with Gfp should be treated with the antibiotic colistin for 20 h. This is done by changing the medium irregating the flow channels of interest to medium containing colistin in a final concentration of 25 μg/ml. Addition of 15 μl Propidium iodide to 1l media (PI, 20mM/ml in DMSO) as Live/Dead indicator. Exchange also the medium in the bubble traps.
Note that PI is light sensitive (-> Cover biofilm system with silver foil).

Day 9 (10/1-11). CLSM image acquisition

Images should be acquired from Biofilm systems I and II (CFP/YFP). All microscopic observations are performed by the use of a Zeiss LSM510 Confocal Laser Scanning Microscope (CLSM) equipped with lasers, filter sets, and detectors for simultaneous monitoring of Cfp (excitation 458nm, emission 480-520nm) and Yfp (excitation 514nm, emission 535-590 nm). The images are acquired with a 40x/1.3 Plan-Neofluar oil objective.
(See also the protocol for biofilm work on the wiki page)

Day 10 (11/1-11). CLSM image acquisition.

All microscopic observations are performed by the use of a Zeiss LSM510 Confocal Laser Scanning Microscope (CLSM) equipped with lasers, filter sets, and detectors for simultaneous monitoring of Gfp (excitation 488nm, emission 517nm) and red-fluorescence emitted from the PI (excitation 488nm, emission 565nm); as well as Cfp (excitation 458nm, emission 480-520nm) and Yfp (excitation 514nm, emission 535-590 nm). The images are acquired with a 40x/1.3 Plan-Neofluar oil objective.

(See also the protocol for biofilm work on the wiki page)

Day 11 (12/1-11). Image analysis.

Prepare images of the 4-day-old biofilms and the colistin treated by the use of the Imaris software.

Questions

The final presentation must include:

How are the wt and the *pilA* mutants spatially distributed in the mixed biofilm? What does this distribution suggest in terms of the role of type IV pili and migration in *P. aeruginosa* biofilm development?

Which parts of the biofilm display tolerance towards colistin? How does this relate to the motile and non-motile subpopulations?

Which parts of the biofilm display the highest growth activity? How does this relate to the motile and non-motile and to the colistin-sensitive and colistin-tolerant subpopulations?

References:

Involvement of bacterial migration in the development of complex multicellular structures in Pseudomonas aeruginosa biofilms. Klausen M, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T. *Mol Microbiol*. 2003 Oct;50(1):61-8

Differentiation and distribution of colistin- and sodium dodecyl sulfate-tolerant cells in Pseudomonas aeruginosa biofilms. Haagensen JA, Klausen M, Ernst RK, Miller SI, Folkesson A, Tolker-Nielsen T, Molin S. *J Bacteriol* 189(1):28-37

Distribution of bacterial growth activity in flow-chamber biofilms. Sternberg C, Christensen BB, Johansen T, Toftgaard Nielsen A, Andersen JB, Givskov M, Molin S. *Appl Environ Microbiol* 1999 Sep;65(9):4108-17.